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Timing of the retinoid-signalling pathway determines the expression of neuronal markers in neural progenitor cells

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Abstract

By culturing neural progenitor cells in the presence of retinoid receptor agonists, we have defined the components of the retinoid-signalling pathway that are important for the birth and maintenance of neuronal cells. We provide evidence that depending on the order and combination of retinoid receptors activated, different neuronal cells are obtained. Astrocytes and oligodendrocytes are predominantly formed in the presence of activated retinoic acid receptor (RAR) α, whereas motoneurons are formed when RARβ is activated. We have looked at the regulation of two transcription factors islet-1/2 which are involved in neuronal development. We find that activated RARβ up-regulates islet-1 expression, whereas activation of RARα can either act in combination with RARβ signalling to maintain islet-1 expression or induce islet-2 expression in the absence of activated RARβ. RARγ cannot directly regulate islet-1/2 but can down-regulate RARβ expression, which results in loss of islet-1 expression. We finally show that activated RARα is one of the final steps required for a mature motoneuron phenotype.

Keywords: Retinoid-signalling pathway; Neuronal markers; Progenitor cells

Introduction

Recently there has been much interest in defining the pathways required to differentiate stem cells into a particular fate, so that the resultant cells can be used to replace damaged or diseased tissue. For example, embryonic stem (ES) cells can be forced to differentiate into macrophages, mast cells or neutrophils by IL-3 (Wiles and Keller, 1991) and muscle cells by transforming growth factor (TGF) (Schuldiner et al., 2000; Slager et al., 1993). Retinoic acid (RA) can cause the differentiation of stem cells into three different lineages; astrocytes, oligodendrocytes and neurons (Arnhold et al., 2000; Jang et al., 2004; Qu et al., 2003; Wohl and Weiss, 1998).

Retinoid signalling is mediated by retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), of which there are three subtypes of each; α, β and γ (Bastien and Rochette-Egly, 2004). Further isoforms of these receptors are obtained by alternative splicing and differential promoter usage (Leid et al., 1992). Transcription occurs when RA binds to an RAR/RXR heterodimer, which then binds to retinoic acid response elements (RAREs), located in the regulatory regions of target genes (Bastien and Rochette-Egly, 2004).

During differentiation of CNS progenitor cells, retinoids can be detected along the length of the neural tube (Colbert et al., 1993; Maden et al., 1998; McCaffery and Drager, 1994; Rossant et al., 1991), indicating the importance of retinoid signalling during this process. The function of RA in neuronal differentiation may be carried out by the activation of different RARs, as during development of the mouse cord, RARβ, RARα and RXRγ are first expressed

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at E10.5 along the entire anterior posterior (AP) axis (Colbert et al., 1995). At E12.5, RARβ becomes restricted to the cervical and lumbar domains while RARα and RXRγ continue to be expressed along the AP axis (Colbert et al., 1995).

Two transcription factors that are involved in neuronal fate are islet-1 and islet-2, which are members of the LIM homeodomain family (Tsuchida et al., 1994). Motoneurons are derived from progenitor cells which all initially express islet-1 (Ericson et al., 1992). In islet-1 null mice, spinal motoneurons do not develop indicating that its expression is critical for motoneuron specification (Pfaff et al., 1996). Five subtypes of motoneurons are then derived from this progenitor population, four of which express islet-2 (Tsuchida et al., 1994). RA signalling has been shown to be involved in the initial induction of islet-1 and the subsequent differentiation of some subtypes of motoneurons (Sharpe and Goldstone, 1997; Sockanathan and Jessell, 1998; Sockanathan et al., 2003), but the RARs involved in these steps are not known.

We have looked at the role of retinoid signalling in the fate of neural progenitor cells (NPCs) and the regulation of two neuronal markers islet-1 and islet-2 by using retinoid receptor-specific agonists. In cultures of NPCs, we show that by activating specific RARs, different neuronal fates can be obtained. Furthermore, depending on the order and combination of activation of retinoid receptors the number of islet-1 and islet-2-expressing neurons are far higher than can be obtained by using RA alone. We finally show that when RARβ signalling is activated in motoneurons, neurite outgrowth is obtained while RARα signalling is one of the final steps in their maturation.

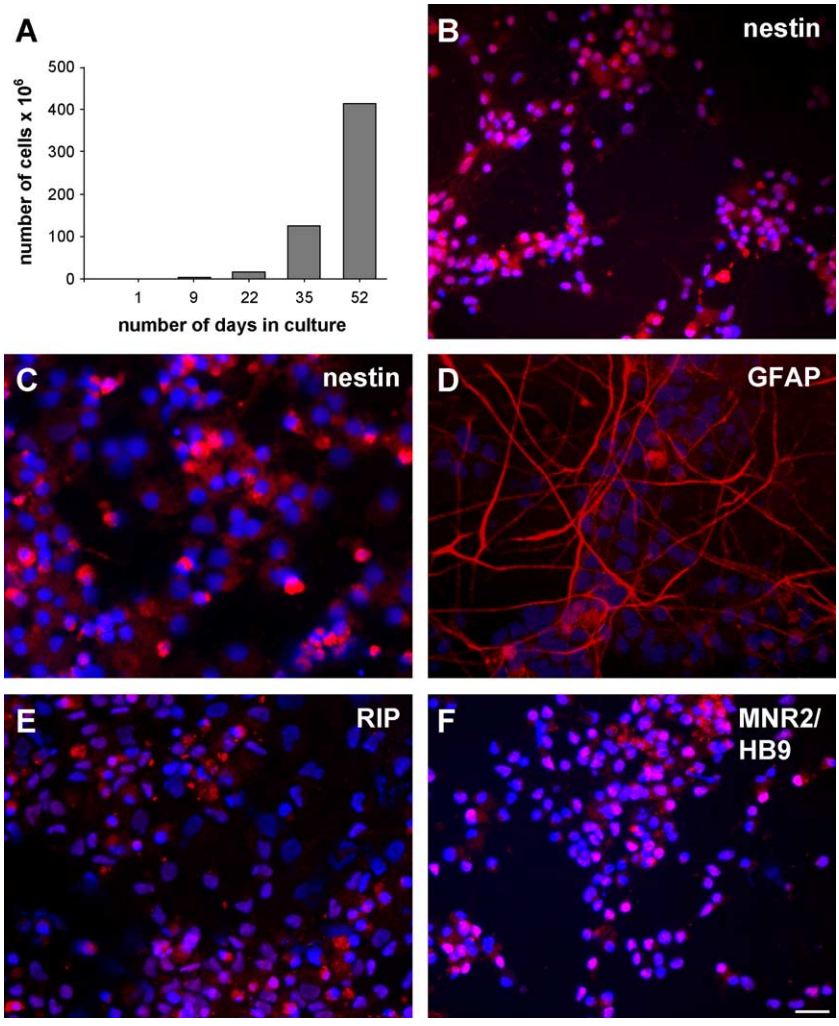

Fig. 1. Expansion of NPCs, isolated from rat E14 spinal cord ventricular layer by FGF-2 and their differentiation into neural cells by RA. (A) Graph of number of NPCs with increasing time, cultured in the presence of 20 ng/ml FGF-2. Merged pictures of DAPI-stained cells (blue) and either nestin, GFAP, RIP or MNR2/HB9-stained cells (red), positive cells for both markers are shown in pink. (B) Expression of nestin in FGF-2 cultured NPCs after 9 days. Expression of neural markers in NPCs cultured for 9 days in FGF-2 and then for 48 h in 0.1 μM RA. (C) Nestin. (D) GFAP. (E) RIP. (F) MNR2/HB9. NPCs can be expanded in vitro by FGF-2 and they express nestin. In contrast, when they are switched to RA, nestin expression is lost and astrocytes (GFAP-positive cells), oligodendrocytes (RIP-positive cells) and motoneurons (MNR2/HB9-positive cells) are formed. Scale bar: 50 μm.

Materials and methods

Culture of neural progenitor cells

The isolation and in vitro propagation of NPCs is based on previously described procedures (Minger et al., 1996). Briefly, the spinal neural tube ventricular zone was removed at gestational day 14 (E14) from Fischer 344 rat embryos (crown-rump length 9–11 mm) and collected in sterile Dulbecco's phosphate buffered saline (PBS). Tissue was incubated in 0.1% trypsin/PBS for 30 min at 37°C, centrifuged at 1000 × *g* and resuspended in PBS-glucose three times, then dissociated to a single cell suspension by repeated pipetting through narrowed Pasteur pipettes. Cell viability and density was determined by trypan blue exclusion and haemocytometric counting. Cells were plated at a density of 30,000 cells per well on 13 mm^2 glass coverslips precoated with 10 μg/ml polyornithine and 10 μg/ml laminin (Gibco) in 24-well plates (Nunc). The cells were grown in DMEM/F12 high glucose media with N2 supplement (Gibco) in 95% air/5% CO2 humidified atmosphere in the presence of 20

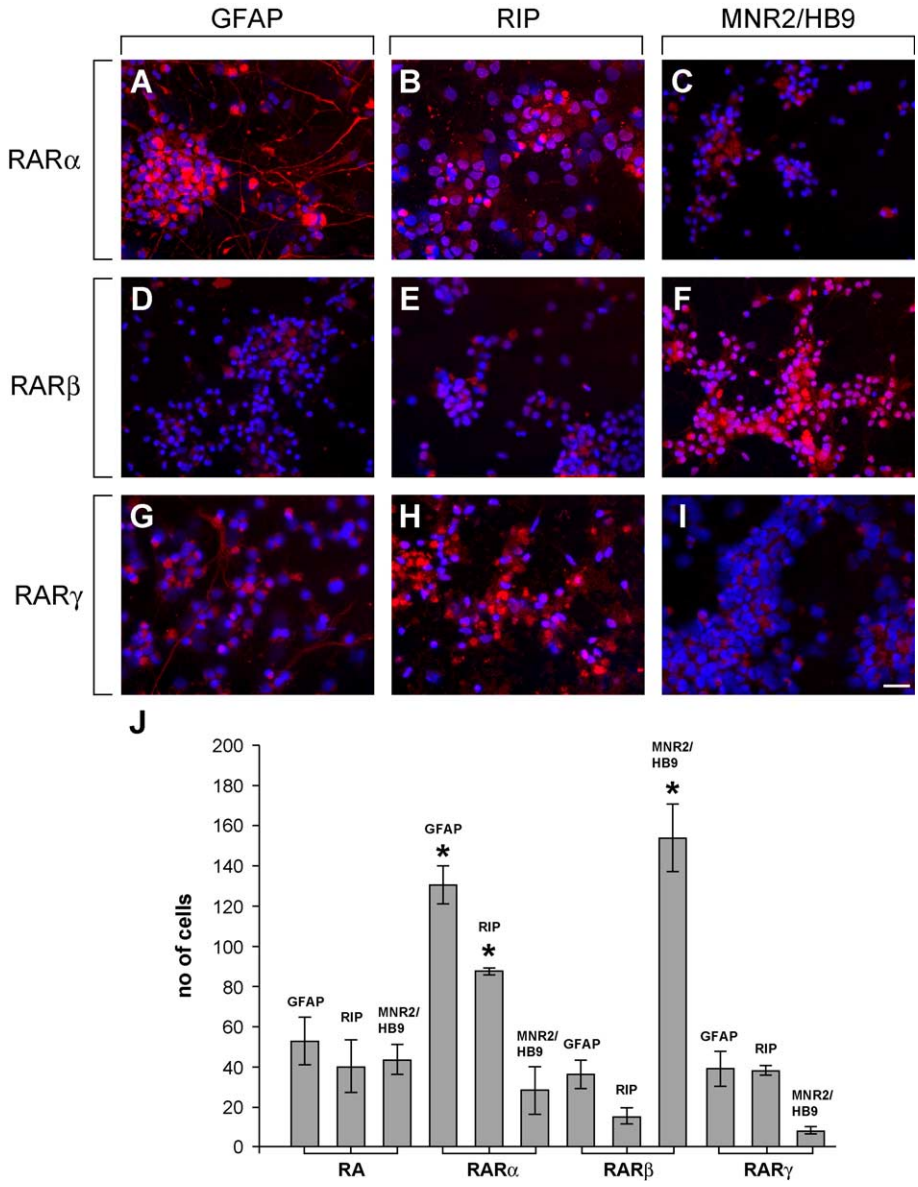

Fig. 2. Effect of retinoid agonists on NPC differentiation. NPCs were cultured for 48 h in 0.1 μM of the following: RARα agonist (A–C); RARβ agonist (D–F); RARγ agonist (G–I). Merged pictures of DAPI-stained cells (blue) and either GFAP, RIP or MNR2/HB9-stained cells (red), positive cells for both markers are shown in pink. (A, D and G) GFAP. (B, E and H) RIP. (C, F and I) MNR2/HB9. (J) Quantification of different types of neural cells in response to retinoid agonists. In the presence of RARα agonist, the majority of the NPCs differentiated into astrocytes (GFAP-positive cells) and oligodendrocytes (RIP-positive cells), which were significantly greater than those formed in RA. In the presence of the RARβ agonist, NPCs mainly differentiated into motoneurons (MNR2/HB9-positive cells), which were significantly greater than those formed in RA. *$P < 0.05$, Student's *t* test. Scale bar: 50 μm.

ng/ml FGF-2 for 72 h. The media was then exchanged and supplemented with 0.1 μM of the appropriate retinoid agonist (Corcoran et al., 2000) instead of FGF-2. The agonists used were all trans RA (Sigma) and retinoid-specific agonists obtained from CIRD Galderma, Sophia-Antipolis, France. These were CD366, which activates RARα; CD2019, which activates RARβ; CD437, which activates RARγ; and CD2809, which activates all RXRs. Culture conditions were carried out three times, using two coverslips per treatment.

In situ hybridisation and immunohistochemistry

Both in situ hybridisation and immunohistochemistry were carried out as previously described (Corcoran et al., 2000). The antibodies used were obtained from the Developmental Studies Hybridoma Bank (University of Iowa) except where stated. These were, anti-mouse islet-1, anti-mouse islet-2, anti-mouse MNR2, anti-rabbit NF200 (Sigma), anti-mouse GFAP (Chemicon), anti-mouse RIP and anti-mouse p75 (Chemicon). MNR2 staining has been used to identify HB9 and/or HB9 related protein in rat tissue (Takahashi and Osumi, 2002). Secondary antibodies used were Cy3 and FITC conjugated (Jackson). The cultures were counterstained with DAPI to detect nuclei. Cultures were analysed at a 100× magnification, images were captured by Image Pro plus software. Neuronal cells were selected for positive signals and either counted or neuronal lengths measured using the computer software. Six random fields of 40 mm^2 of the final magnification were taken per coverslip. Graphs were plotted and statistical analysis carried out using sigma-plot software. Histograms show mean ± SEM of either positive neurons/field or average length of neurites (μm).

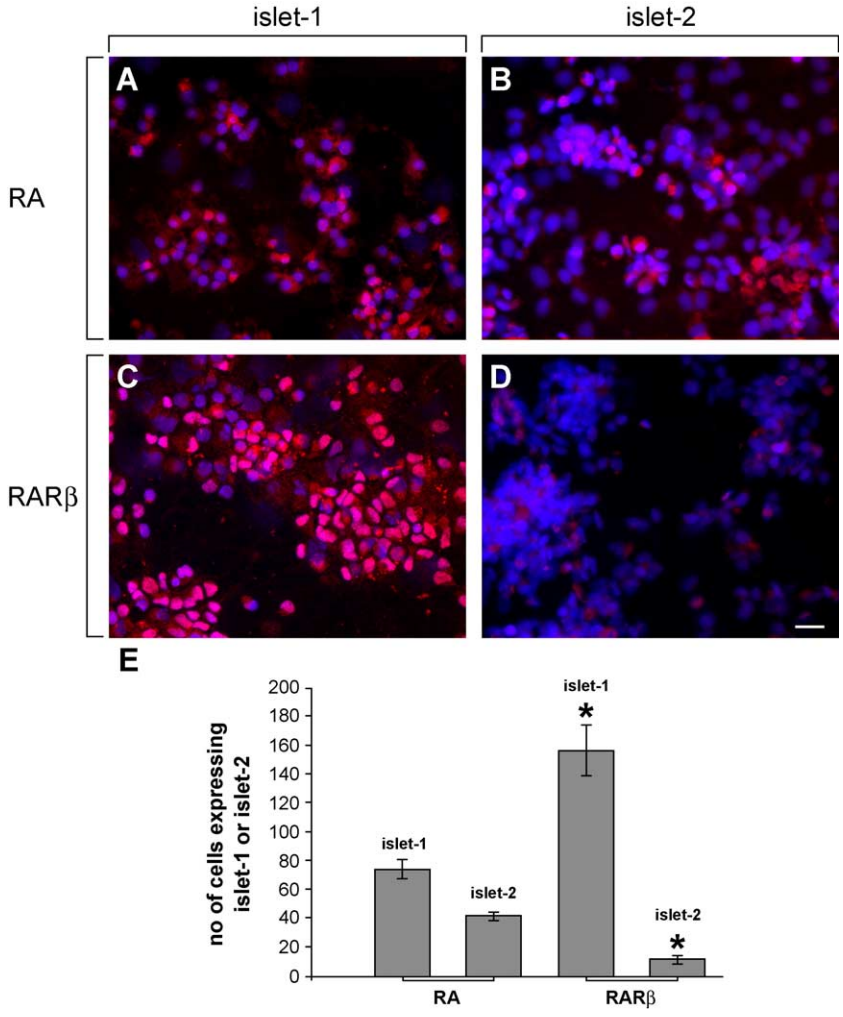

Fig. 3. Effect of RA or RARβ agonist on NPC islet-1 and islet-2 expression. NPCs were cultured for 48 h in of 0.1 μM of the following: RA (A–B); RARβ agonist (C–D). Merged pictures of DAPI-stained cells (blue) and either islet-1 or islet-2-stained cells (red), positive cells for both markers are shown in pink. (A) (C) Islet-1. (B and D) Islet-2. (E) Quantification of islet-1 and islet-2 cells in response to retinoid agonists. In the presence of RARβ agonist, significantly more islet-1 motoneurons are formed than in RA-treated cultures. In contrast, the number of islet-2 motoneurons is significantly less in the RARβ agonist than RA-treated cultures. *$P < 0.05$, Student's t test. Scale bar: 50 μm.

Results

NPCs can be isolated from the rat ventricular zone

We first asked if cells isolated from the rat E14 spinal cord ventricular layer have the characteristics of NPCs. These are as follows: firstly, they undergo self-renewal; secondly, they express nestin during this renewal process; and thirdly, they differentiate into various types of cells of the nervous system (Cai et al., 2002; Gage et al., 1995; Maric and Barker, 2004; Minger et al., 1996). The cells were grown in the presence of the mitogen FGF-2 (20 ng/ml) and they were passaged and counted at 1, 9, 22, 35 and 52 days of culture. They were assessed for neurogenic potential by taking cells at each passage point and culturing them in the presence of 0.10 μM RA for 48 h instead of FGF-2. With increasing time, the number of cultured cells increased from the seeding density of 0.1 million cells/ml to over 400 million cells/ml at 52 days of culture, which was the end point of the experiment (Fig. 1A). Most, if not all the cells, were positive for nestin at each passage point (Fig. 1B). After RA treatment, we found that few, if any, nestin-positive cells remained (Fig. 1C) and three different neural cell types could be obtained. These were astrocytes as shown by GFAP staining (Fig. 1D), oligodendrocytes as shown by RIP staining (Fig. 1E) and motoneurons as shown by MNR2/HB9 staining (Fig. 1F).

RA can activate all three RARs; therefore, we next asked if by activating the RARs individually, particular types of neural cells could be obtained. We used the following receptor-specific agonists: CD366, which activates RARα; CD2019, which activates RARβ; and CD437, which activates RARγ. The NPCs were cultured for 48 h in FGF-2 and then in 0.10 μM of retinoid agonist, either RARα, RARβ or RARγ for a further 48 h. In the presence of RARα agonist, the majority of cells were astrocytes (Figs. 2A and J) and oligodendrocytes (Figs. 2B and J), which were significantly greater than those formed in the presence of RA, but there were few motoneurons (Figs. 2C and J). In contrast, in the presence of RARβ agonist, the majority of the cells were motoneurons (Figs. 2F and J), which were significantly greater than those formed in the presence of RA, but there were few astrocytes (Figs. 2D and J) or oligodendrocytes (Figs. 2E and J). In the presence of the RARγ agonist, few, if any, motoneurons could be detected (Figs. 2I and J) and only some astrocytes and oligodendrocytes (Figs. 2G and H), but these were not significantly different to those formed in the presence of RA (Fig. 2J). These data indicate that the RAR receptor pathways have different roles in the determination of NPCs.

Activation of RARβ is required for islet-1 expression

As the majority of motoneurons occur in the RARβ agonist-treated cultures, we next asked if RARβ, like RA, could regulate islet-1 and islet-2, which are involved in the determination of motoneurons (Sharpe and Goldstone, 1997; Sockanathan and Jessell, 1998; Sockanathan et al.,

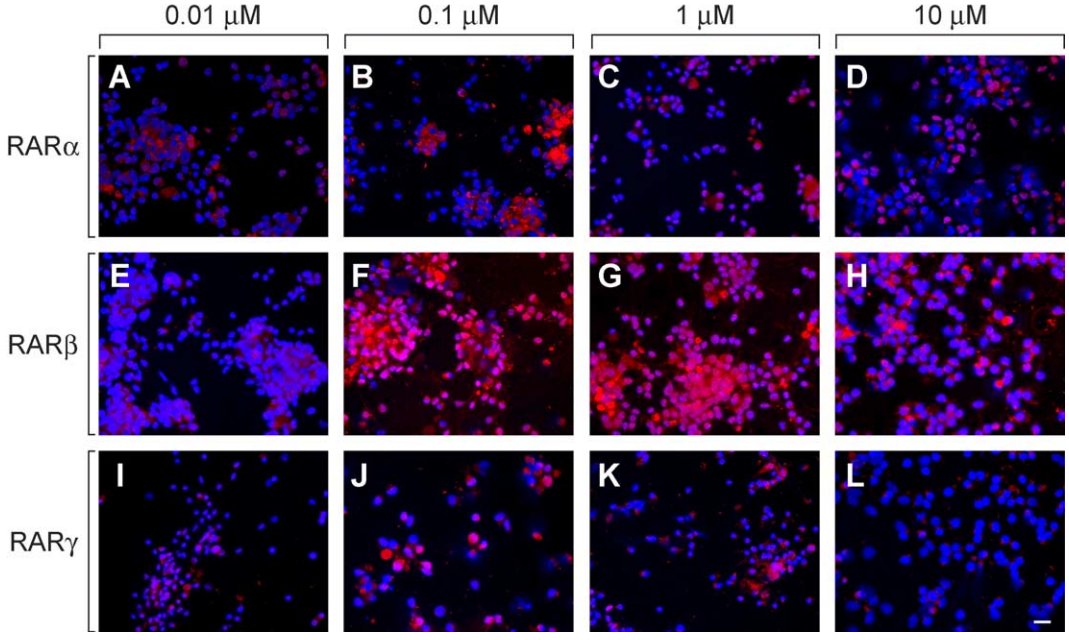

Fig. 4. Effect of increasing doses of retinoid agonists on the number of islet-1-expressing neurons. NPCs were cultured in FGF-2 for 48 h then were switched to 0.01 μM (A, E and I), 0.1 μM (B, F and J), 1 μM (C, G and K) or 10 μM (D, H and L) of either RARα, β or γ agonist for a further 48 h. Merged pictures of DAPI-stained cells (blue) and islet-1-stained cells (red), positive cells for both markers are shown in pink. (A–D) RARα agonist. (E–H) RARβ agonist. (I–L) RARγ agonist. In the presence of RARα or γ agonist, very few islet-1 cells were obtained at any dose; while in the presence of RARβ agonist, islet-1-expressing cells were predominantly formed in 0.1 and 10 μM of the agonist. Similar data were obtained when the cells were cultured in the presence of the agonists for 4 days. Scale bar: 50 μm.

2003). In the presence of both retinoids, islet-1-expressing cells were obtained (Figs. 3A and C); however, their numbers were significantly higher in the presence of RARβ agonist than RA (Fig. 3E). In contrast, while islet-2-expressing cells were obtained with both retinoids (Figs. 3B and D), there were significantly less in RARβ agonist than in the RA-treated cultures (Fig. 3E).

In order to rule out the possibility that the RARβ agonist can activate other RARs, and or the other retinoid agonists can activate RARβ at particular concentrations, we asked if islet-1-expressing cells could be induced in the NPC cultures with increasing concentrations of the agonists. The NPCs were cultured in FGF-2 for 48 h then were switched to 0.01, 0.1, 1, 10 or 100 μM of either RARα, β or γ agonist for a further 48 h. In the presence of the RARα agonist, very few islet-1-positive cells were obtained and these did not increase with increasing dose of agonist (Figs. 4A–D). Similar data were obtained with the NPCs treated with RARγ agonist (Figs. 4I–L). In NPCs cultured in RARβ agonist, islet-1-expressing cells were predominantly formed in the presence of 0.1 and 10 μM of the agonist (Figs. 4F and G) but not in either 0.01 or 100 μM (Figs. 4E and H). Similar data were obtained when the NPCs were cultured in the presence of the agonists for 4 days rather than 48 h (data not shown). These data suggest that the activation of RARβ is responsible for the majority of islet-1 expression in the NPCs and that the other receptor agonists can neither cause a significant increase in this expression, nor activate RARβ.

RARα activation maintains islet-1 expression in the presence of RARβ activation but induces islet-2 expression in its absence

Previous work has shown that further retinoid signalling via RARs occurs to determine the fate of motoneurons once the islet-1 pool is formed (Sockanathan et al.,

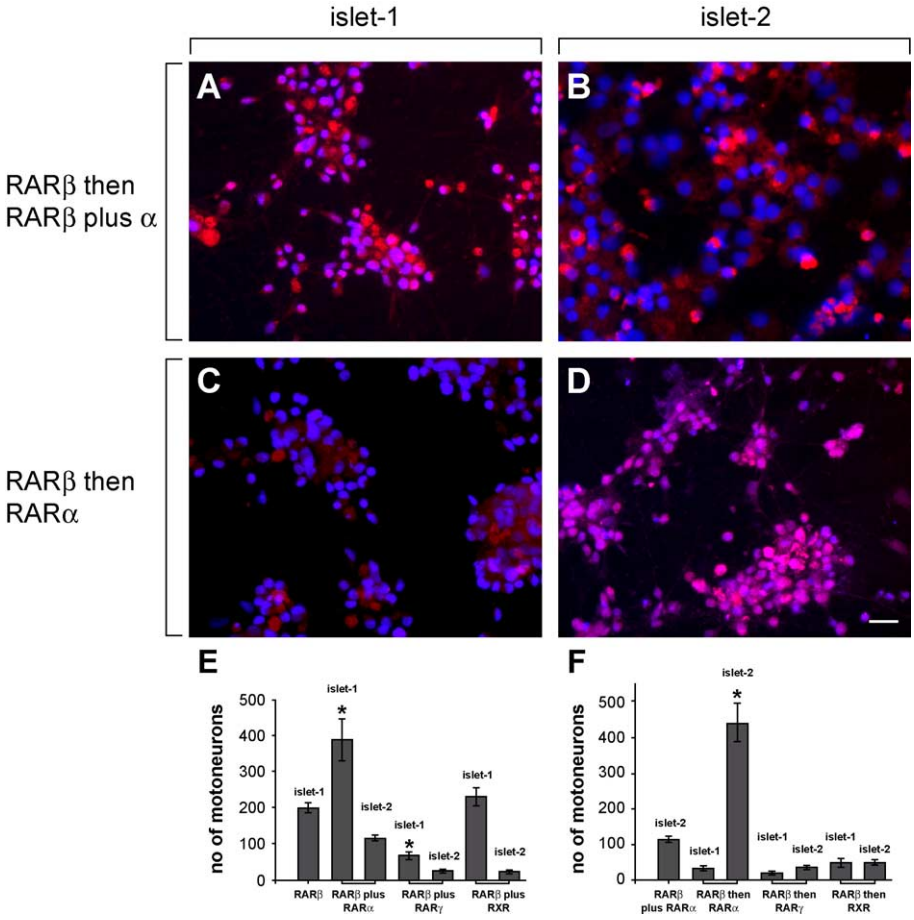

Fig. 5. Effect of combinational or sequential activation of retinoid receptors on NPC islet-1 and islet-2 expression. NPCs were cultured for 48 h in 0.1 μM of RARβ agonist. They were then cultured for 48 h in a second retinoid agonist (0.1 μM), either in the presence or absence of the RARβ agonist. Merged pictures of DAPI-stained cells (blue) and either islet-1- or islet-2-stained cells (red), positive cells for both markers are shown in pink. (A and C) Islet-1. (B and D) Islet-2. Quantification of number of islet-1- and islet-2-expressing motoneurons in response to the retinoid agonists. (E) In the presence of RARβ agonist, (F) in the absence of RARβ agonist. In RARβ plus RARα agonist-stimulated cultures, there is a significant increase in the number of islet-1-expressing motoneurons compared to the RARβ agonist-stimulated cultures; whereas in RARβ plus RARγ-treated cultures, there is a significant loss in the number of islet-1-expressing motoneurons compared to the RARβ agonist-stimulated cultures. While few islet-2 motoneurons were obtained, the majority were in the RARβ plus RARα cultures compared to the other agonist-treated cultures. In the RARβ then RARα agonist-stimulated cultures, there is a significant increase in the number of islet-2-expressing motoneurons compared to RARβ plus RARα-treated cultures $^{*}P < 0.05$, Student's t test. Scale bar: 50 μm.

2003) and four of the resultant subtypes of motoneurons express islet-2. This retinoid signalling may also be mediated by RXRs, as it has been shown to be involved in the maturation of limb-innervating motor neurons (Solomin et al., 1998). Therefore, we next asked what effect activating either RARα, γ or RXR has on islet-1/2 expression. NPCs were cultured in FGF-2 containing medium for 48 h followed by 0.10 μM RARβ agonist for 48 h in order to form islet-1-expressing motoneurons, and then in 0.10 μM of RARα, γ or a pan RXR agonist (CD2809) for 48 h in the presence or absence of 0.10 μM RARβ agonist. The majority of islet-1 neurons were formed in the RARβ plus RARα-treated cultures (Fig. 5A), and these were significantly greater than in the RARβ agonist-treated cultures while the converse was true in RARβ plus RARγ-treated cultures (Fig. 5E). In the RARβ plus RXR-treated cultures, their numbers were not significantly different compared to the RARβ-stimulated cultures (Fig. 5E).

In contrast, few if any islet-2-expressing neurons could be formed with any of the agonists when RARβ agonist was present (Figs. 5B and E). However, in the RARβ then RARα agonist-treated cultures, there was a loss of islet-1-expressing neurons (Fig. 5C) and an increase in islet-2-expressing neurons (Fig. 5D), which were significantly greater compared to the combined activation of RARβ plus RARα (Fig. 5F). In the RARβ then RARγ-treated cultures and RARβ then RXR-treated cultures, islet-1-expressing neurons were lost (Fig. 5F) and there were some islet-2-expressing neurons; however, there was no significant difference in their numbers compared to RARβ plus RARα-treated cultures (Fig. 5F). These data indicate that the loss of RARβ activation leads to the loss of islet-1 expression and activation of RARα induces islet-2 expression.

Next, we asked if any of the RARs could regulate one another as has been previously shown (Corcoran et al., 2000; Ferrari et al., 1998; Matt et al., 2003). In RARβ plus RARα agonist-treated cultures, all three RARs could be detected by in situ hybridisation (Figs. 6A–C); similar data were obtained in the RARβ plus pan RXR agonist-treated cultures (Figs. 6G–I). In contrast, in the RARβ plus RARγ agonist-treated cultures, there was a loss of RARβ expression (Fig. 6E) while RARα and γ expression were maintained (Figs. 6D and F). This suggests that the RARγ agonist can inhibit RARβ expression and as a consequence there is a loss of islet-1 expression.

RARα signalling is required to maintain a mature motoneuron phenotype

The above data suggest that motoneurons must initially activate RARβ signalling in order to induce islet-1, whereas previously it has been shown that adult motoneurons express RARα rather than RARβ (Corcoran et al., 2002). Mature motoneurons express the low-affinity neurotrophin receptor (p75) (Camu and Henderson, 1992) and they extend neurites containing neurofilaments (Xu et al., 1994). Therefore, we asked if activation of the RARα-signalling pathway could give rise to p75-expressing motoneurons and under what treatments neurites could be formed. In NPC cultures treated with RARβ plus either RARα, γ or the pan RXR agonist, very little p75

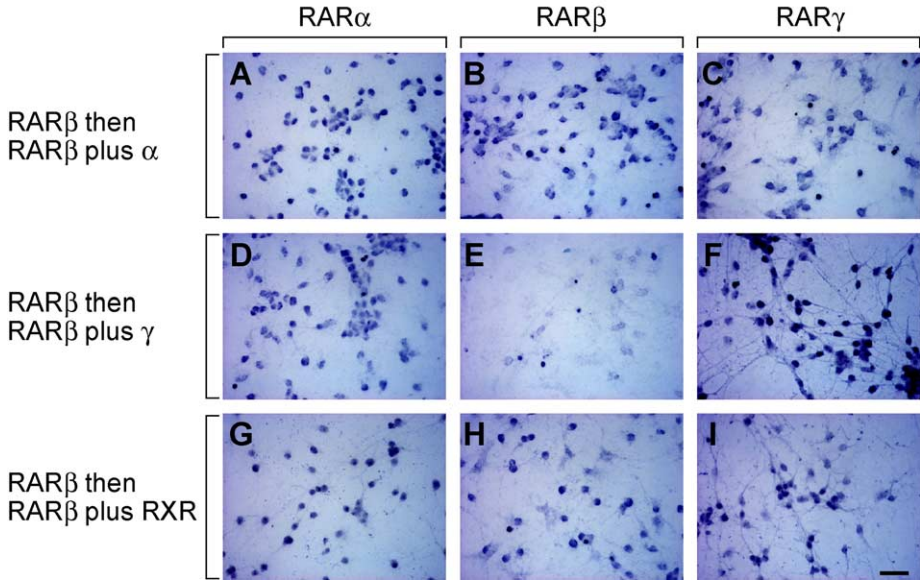

Fig. 6. Expression of RARs in NPCs. Effect of the combinational activation of retinoid receptors in NPCs on expression of RARs. NPCs were cultured for 48 h in 0.1 μM of RARβ agonist. They were then cultured for 48 h in 0.1 μM of the following: RARβ plus RARα agonist (A–C); RARβ plus RARγ agonist (D–F); RARβ plus pan RXR agonist (G–I). In situ hybridisation of the following: (A, D and G) RARα, (B, E and H) RARβ, (C, F and I) RARγ. In RARβ plus RARγ-treated cultures, there is a loss of RARβ expression. Scale bar: 50 μm.

immunoreactivity was detected (Figs. 7A and E). In contrast, in NPC cultures treated with RARβ then RARα, γ or RXR agonist, more p75 motoneurons were formed than in the presence of RARβ and a second retinoid agonist (Fig. 7E). The majority were formed in the RARβ then RARα agonist-treated cultures (Fig. 7C) and were significantly greater than in any of the other culture conditions (Fig. 7E). This suggests that the absence of RARβ signalling leads to a mature motoneuron phenotype and that RARα signalling can maintain it.

Neurites were formed in all six culture conditions the longest in the RARβ plus RARα (Figs. 7B and F) and RARβ then RARα agonist-treated cultures (Figs. 7D and F). In the RARβ plus RARγ, RARβ then RARγ and RARβ then RXR agonist-treated cultures, neurite lengths were significantly shorter than RARβ plus RARα-treated cultures (Fig. 7F). There was no significant difference in neurite lengths between RARβ plus RXR and RARβ plus RARα-treated cultures (Fig. 7F).

Discussion

The CNS is thought to develop from self-renewing stem cells that can generate the three major cell types: oligodendrocytes, astrocytes and neurons (Gage, 2000). We have shown here that cells obtained from the rat E14 spinal cord ventricular layer have the characteristics of NSCs. They can be expanded in vitro by the mitogen FGF-2 and can differentiate in the presence of RA into the three cell types of the CNS. While RA can activate all the RARs, we have shown by using RAR-specific agonists that RARα signalling predominantly causes the differentiation of NPCs into astrocytes and oligodendrocytes and some are formed in the presence of RARγ signalling. In the presence of RARβ signalling, NPCs differentiate into motoneurons (Fig. 8). This increase in neuronal differentiation by RA has also been shown in vivo. The addition of RA to cultured chicken neural tubes (Sockanathan and Jessell, 1998) and over-expression of retinoid receptors in Xenopus embryos causes

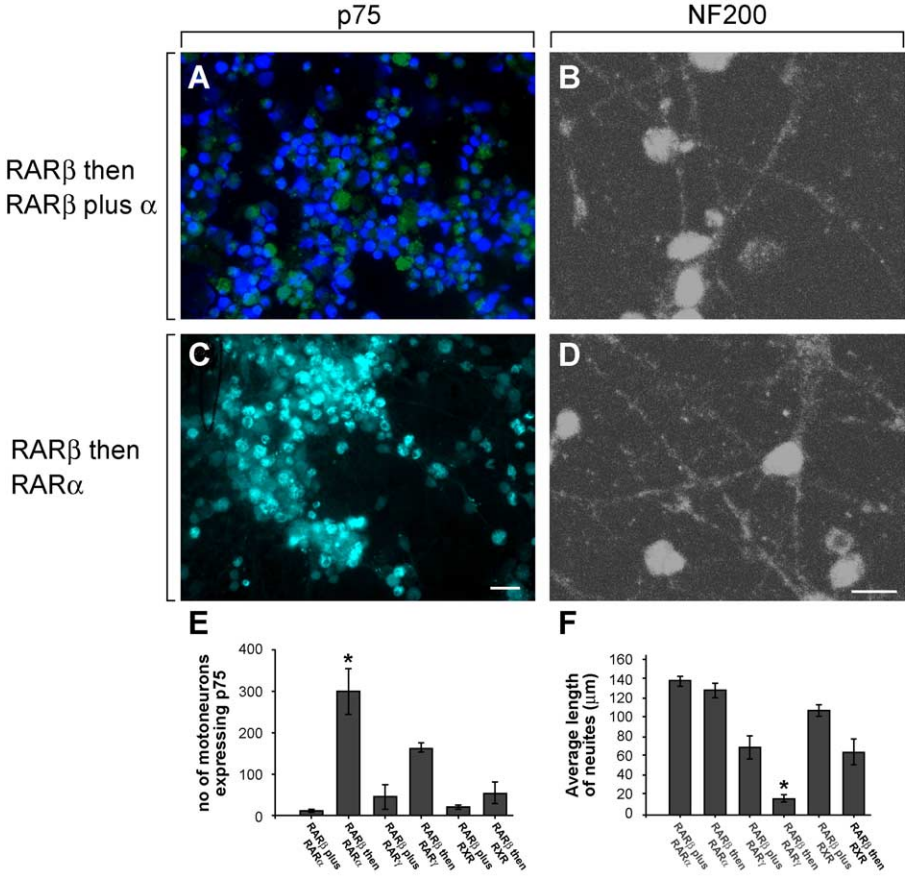

Fig. 7. Effect of retinoid agonists on mature phenotype of motoneurons. NPCs were cultured for 48 h in 0.1 μM of RARβ agonist. They were then cultured for 48 h in a second retinoid agonist (0.1 μM), either in the presence or absence of the RARβ agonist. Merged pictures of DAPI-stained cells (blue) and p75-stained cells (green), positive cells for both markers are shown in turquoise. (A) RARβ plus RARα agonist. (C) RARβ then RARα agonist. Immunohistochemistry of NF200 in (B) RARβ plus RARα agonist and (D) RARβ then RARα agonist. (E) Quantification of number of p75 motoneurons in retinoid agonist-treated cultures. The majority of p75 neurons are formed in RARβ then RARα agonist, and RARβ then RARγ agonist-stimulated cultures, and there is a significant difference between these two treatments. (F) Quantification of neurite lengths (μm) in retinoid agonist-treated cultures. The longest neurite lengths were obtained in the RARβ plus RARα, and RARβ then RARα agonist-treated cultures, significantly less neurite outgrowth was obtained in the other agonist treated. *$P < 0.05$, Student's t test. Scale bar: 50 μm in panels A and C, 10 μm in panels B and D.

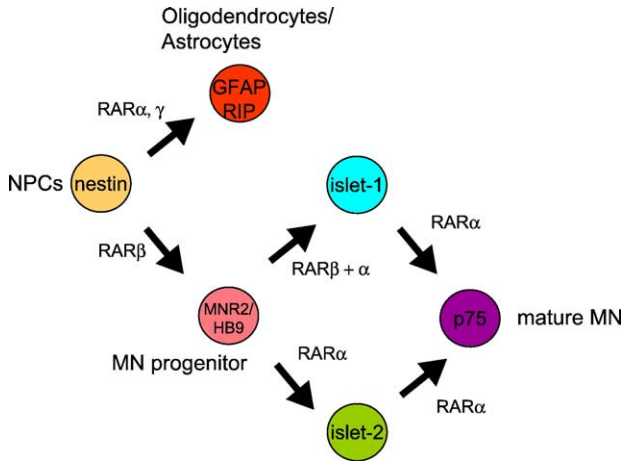

Fig. 8. Model of NPC differentiation in response to retinoid agonists. NPCs differentiate into oligodendrocytes/astrocytes in the presence of RARα and γ agonists, the majority being formed in the former. NPCs mainly differentiate into motoneurons in the presence of RARβ agonist. Once the motoneuron progenitor is formed, RARα in combination with RARβ agonist can maintain islet-1 expression or in its absence can induce islet-2 expression. In the presence of RARα agonist but absence of RARβ agonist, mature motoneurons-expressing p75 are obtained.

an increase in the number of neurons (Sharpe and Goldstone, 1997).

We have not shown that the cells are true stem cells, in that we have not taken a single cell and derived multiple lineages from it; such experiments are difficult to do since single cells do not survive well in culture. We may therefore have isolated a heterogeneous population of cells that can give rise to several lineages. In support of this is the fact that in the spinal cord astrocytes are derived from the dorsal region of the cord (Lu et al., 2002; Pringle et al., 1998; Zhou and Anderson, 2002), while oligodendrocytes and motoneurons are derived from the ventral region (Hall et al., 1996; Novitch et al., 2001; Pringle et al., 2003). On the other hand, it has been shown that neurospheres isolated from the same region and stage of the cord that we have used do have characteristics of NSCs; however, this may be due to their expansion in vitro and may not represent the in vivo situation (Gabay et al., 2003).

Generation of islet-1/2-expressing neurons by the RARs

In order to form mature motoneurons, further retinoid signalling is required. Previous work has shown in vivo that overexpression of a constitutive active retinoid receptor in thoracic motoneurons prevents their differentiation and leads to neuronal death; whereas blocking retinoid signalling in brachial motoneurons inhibits the differentiation of medial lateral motor column neuron, which expresses islet-2 and lim-1, and forces them to become Column of Terni neurons, which express islet-1, and lateral median motor column neurons, which express islet-1/2 (Sockanathan et al., 2003).

We have shown here that specific RAR receptor pathways are involved in postmitotic neuronal differentiation and that the combinational actions of RAR-signalling control islet-1/2 expression. The initial RA-signalling step for motoneuron differentiation is the activation of RARβ (Fig. 8), which induces islet-1, but not islet-2 expression. Neither RARα nor γ activation can induce islet-1 expression to the same levels as RARβ activation, even with increasing concentrations. It is unlikely that this is due to a timing issue, as when the NPCs were cultured for 4 days in either RARα or γ agonists, islet-1 expression was still not induced to any appreciable extent. This suggests that these signalling pathways cannot substitute for RARβ signalling in the initial specification of the motoneurons. However, once this step occurs, then signalling by RARα and γ is required for further maturation of the motoneurons. Activated RARα signalling can maintain islet-1 expression in combination with activated RARβ (Fig. 8). When RARα is activated either in the presence or absence of RARβ signalling, then islet-2-expressing neurons are obtained (Fig. 8). However, it is only when RARβ expression is down-regulated that there is a dramatic increase in the number of islet-2 neurons produced by RARα signalling. The loss of RARβ expression may be due to activated RARγ, as this receptor is able to down-regulate RARβ. It has also previously been shown that activation of RARβ is antagonistic to RARα and γ activation (Matt et al., 2003), and this may be one reason why we see so few islet-2-expressing neurons in the presence of RARβ plus RARα agonists. Therefore, while RARα and γ do not play a major role in the initial expression of islet-1, they are important for the regulation of islet-2 expression. We have not identified a role for RXR signalling in the regulation of islet/2, although it is thought to be involved in motoneuron maturation (Solomin et al., 1998). It may be important for the regulation of other LIM homeodomain proteins, known to be involved in motoneuron specification, these include Lim-1 and Lim-3 (Tsuchida et al., 1994).

RARα signalling is required to maintain a mature motoneuron phenotype

One of the final steps in motoneuron maturation is the activation of RARα signalling (Fig. 8). RARβ signalling prevents a mature phenotype occurring, as when this receptor pathway is activated, few if any motoneurons express the p75 receptor. In vivo, it has also been shown that during neurogenesis of the spinal cord, both RARα and RARβ correlate with this process (Colbert et al., 1995; Yamagata et al., 1994), and at its completion RARβ expression is down-regulated while RARα expression is maintained (Corcoran et al., 2002; Yamagata et al., 1994). There is an increase in p75 expression in the absence of RARβ signalling in the RARα, γ and RXR agonist-treated cultures. However, it is only when the RARα pathway is activated that there is a significant increase in p75-expressing motoneurons compared to any of the other

treatments. This suggests that RARα pathway is required to maintain a mature motoneuron phenotype rather than to induce it (Fig. 8). Previous work has shown that RA signalling is involved in neurite outgrowth (Quinn and De Boni, 1991), and that it occurs via activation of RARβ signalling (Corcoran et al., 2000). We have further shown here that neurite outgrowth occurs in the presence of RARβ signalling and that in its absence only RARα signalling can maintain this outgrowth. This again shows the importance of RARα signalling in the maintenance of a mature motoneuron phenotype.

Our data, showing that the order of activation of retinoid receptors leads to the differentiation of NPCs into various neural cell types, may now have implications for stem cell therapy. One major problem that has to be overcome before stem cells can be used therapeutically is to generate large numbers of homogenous cells. We have shown here that by delineating the retinoid-signalling pathways required for various neural types, far more cells of a particular lineage can be obtained than by using RA alone. One reason why this may occur is because RA is able to activate all retinoid-signalling pathways in the NPCs at the same time. By using retinoid agonists as illustrated here, the retinoid pathways can be activated in a sequential manner as in vivo, this leads to a far greater number of differentiated cells of one particular phenotype.

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